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Antibiofilm Activity of Invasive Plants against *Candida albicans*: Focus on *Baccharis halimifolia* Essential Oil and Its Compounds

Sufi Desrini,^[a, b, c] Julien Ducloux,^[c] Guillaume Hamion,^[c] Charles Bodet,^[d] Jérôme Labanowski,^[e] Mustofa Mustofa,^[f, h] Titik Nuryastuti,^[g, h] Christine Imbert,^[c] and Marion Girardot^{*[c]}

The extracts of five invasive plants were investigated for antifungal and antibiofilm activities against *Candida albicans*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*. The antifungal activity was evaluated using the microdilution assay and the antibiofilm effect by measurement of the metabolic activity. Ethanol and ethanol-water extracts of *Reynoutria japonica* leaves inhibited 50% of planktonic cells at 250 $\mu\text{g mL}^{-1}$ and 15.6 $\mu\text{g mL}^{-1}$, respectively. Ethanol and ethanol-water extracts of *Baccharis halimifolia* inhibited > 75% of the mature biofilm of *C. albicans* at 500 $\mu\text{g mL}^{-1}$. The essential oil (EO) of *B. halimifolia* leaves was the most active (50% inhibition (IC_{50}) at 4 and 74 $\mu\text{g mL}^{-1}$ against the maturation phase and 24 h old-biofilms

of *C. albicans*, respectively). Oxygenated sesquiterpenes were the primary contents in this EO (62.02%), with β -caryophyllene oxide as the major component (37%). Aromadendrene oxide-(2), β -caryophyllene oxide, and ($\pm$)- β -pinene displayed significant activities against the maturation phase (IC_{50} = 9–310 $\mu\text{mol L}^{-1}$) and preformed 24 h-biofilm (IC_{50} = 38–630 $\mu\text{mol L}^{-1}$) of *C. albicans* with very low cytotoxicity for the first two compounds. *C. albicans* remained the most susceptible species to this EO and its components. This study highlighted for the first time the antibiofilm potential of *B. halimifolia*, its EO and some of its components.

Introduction

Candida genus is the most prominent etiology of human fungal infections. As a polyphyletic group of the order Saccharomycotina, *Candida* lives as commensals on healthy human mucous membranes.^[1] However, some species can become pathogenic, especially during changes in their environment, for instance a change in pH, in the immune response, or in the case of a dysbiosis. These changes lead to candidiasis, including candidemia and invasive *Candida* infections with a high risk of morbidity and mortality.^[2] In Europe, the incidence of candidemia was estimated to be about 79 cases per day, of which 29 patients might have a disastrous outcome on Day 30.^[3]

The most common cause of candidemia is *C. albicans*, representing 35% to 60% of isolates. However, many studies recently suggested an increasing incidence of non *albicans* *Candida* (NAC) infections. Accordingly *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei* are the most common species identified in culture.^[4] The pathogenicity of the *Candida* genus is in the link with its capacity to form a biofilm. Indeed, it can build on biotic (mucosa, other microbial cells) or abiotic (medical devices such as catheters, stents, pacemakers, etc.) surfaces of well-constructed biofilms constituted of multiple cells wrapped in an extracellular matrix.^[5] *Candida* spp. biofilms are able to counter the conventional antifungal agents used at concentrations higher than those that reduce planktonic cells. This is due to their complexity and density with various morphotypes encircled by extracellular matrix.^[6] Therefore, the treatment of biofilm-related infections becomes difficult as common drug therapies cannot eradicate *Candida* spp. biofilms.

[a] Dr. S. Desrini
Department of Pharmacology, Faculty of Medicine, Universitas Islam Indonesia, 55584, Yogyakarta Indonesia

[b] Dr. S. Desrini
Doctoral Programme of Faculty Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia

[c] Dr. S. Desrini, J. Ducloux, G. Hamion, Prof. C. Imbert, Dr. M. Girardot
Laboratoire Ecologie et Biologie des Interactions – UMR CNRS 7267, Université de Poitiers, Poitiers, France
E-mail: marion.girardot@univ-poitiers.fr
Homepage: <https://ebi.labo.univ-poitiers.fr/>

[d] Prof. C. Bodet
Laboratoire Inflammation, Tissus Epithéliaux et Cytokines UR 15560, Université de Poitiers, Poitiers, France

[e] Dr. J. Labanowski
IC2MP – UMR CNRS 7285, Université de Poitiers, Poitiers, France

[f] Prof. M. Mustofa
Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia

[g] Prof. T. Nuryastuti
Department of Microbiology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia

[h] Prof. M. Mustofa, Prof. T. Nuryastuti
Indonesia Biofilm Research Collaboration Center UGM-BRIN, Yogyakarta, Indonesia

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Nevertheless, removal and replacement of medical devices are expensive, and may not be feasible for all patients and not necessarily effective. Thus, it reflects the relevance of paying close attention to the search for new molecule drugs targeting *Candida* biofilms with low toxicity levels.

Nature indeed supplies an abundance of plants with many advantages for human beings. The utilization of plants to treat diseases is as old as human territory. As yet, 35,000–70,000 plant species have been appraised for their medicinal purpose.^[7] Unfortunately, related to this approach, the utilization of plants in medicine poses a high risk of overharvesting as well as destructing their habitat.^[8] In Europe, 90% of 1300 medicinal plants are harvested from wild nature.^[9] In this sense, the utilization of invasive plants can be a good point for screening molecule drugs from natural sources without causing the loss of native medicinal plants. Invasive plants are defined as “species that are non-native to a specified geographic area, were introduced by humans (intentionally or unintentionally), and do or can cause environmental or economic harm or harm to humans”.^[10] It is known that invasive alien plants can affect the native species’ richness, their abundance, and the genetic composition of native populations. They also change the ecosystem functioning and the delivery of ecosystem services by altering for instance nutrient, hydrology, or habitat structure. However, they are also a source of promising compounds with great interest in human health.^[11] Several studies reported antimicrobial properties of invasive alien plants such as that of Poljuha and collaborators who described antimicrobial effects of extracts of invasive *Ailanthus altissima* against *Escherichia coli* and *C. albicans*.^[12] The same researchers recently reported the antibacterial and antifungal activities of extracts obtained from six invasive plants present in Croatia (*Ailanthus altissima*, *Ambrosia artemisiifolia*, *Conyza canadensis*, *Dittrichia viscosa*, *Erigeron annuus*, and *Xanthium strumarium*).^[13] Meela et al. reported a great antifungal effect of the invasive *Passiflora suberosa* with an average minimum inhibitory concentration (MIC) of 0.09 mg mL⁻¹ against eight plant fungal pathogens (*Rhizoctonia solani*, *Fusarium oxysporium*, *Penicillium janthinelum*, *Penicillium expansum*, *Aspergillus parasiticus*, *Aspergillus niger*, *Pythium ultimum*, and *Phytophthora nicotianae*).^[14] It is proven that the production of antimicrobial compounds by their specialized metabolism serves as a natural protective mechanism and increase their competitiveness.^[13]

In this context, five invasive plants were chosen for this study and screened for antifungal potential as well as antibiofilm activity against *Candida* species for the first time: *Reynoutria japonica*, *Ambrosia artemisiifolia*, *Buddleja davidii*, *Robinia pseudoacacia*, and *Baccharis halimifolia*.^[15] This study could help to find new compounds as future drug candidates to eradicate *Candida* biofilms. In addition, this study contributes to a better understanding of the pharmacological properties of major invasive plants of France.

Results and Discussion

Plant extracts

Five invasive plants were extracted with four solvents of various polarities (hexane, ethyl acetate, ethanol, and ethanol-water (1:1)). Thirty-two extracts were obtained. The yields were reported in Table S1.

The best total yields were obtained with *B. halimifolia* leaves and stems (63.8 and 58%, respectively). *B. davidii*, *R. japonica*, and *R. pseudoacacia* contained few compounds extractable by the solvents used (3.2–10.7%). By comparing the extracted parts within the same plant, the leaves seemed to contain more extractable molecules than the stems (*B. davidii* 7.2 vs. 3.5%; *R. japonica* 10.7 vs. 3.4%, and *B. halimifolia* 63.8 vs. 58%). Naidoo and collaborators observed similar results when studying *Tabernaemontana ventricosa* stems and leaves’ extracts.^[16] This difference may be explained by the fact that leaves are photosynthetic organs and they produce high concentrations of metabolites.^[17] For the five studied plants, the extractions with the most polar solvents: EtOH (for *B. halimifolia* leaves) or a mixture EtOH-H₂O (for the other tested samples) showed the best yields (1.77–41.4%). This was previously observed in several studies and can be explained by the presence of numerous polar primary and secondary metabolites in plants.^[18]

Antifungal and antibiofilm activities of extracts

The antifungal activity of the 32 extracts was evaluated against planktonic cells of *C. albicans* ATCC 28367. All plants, except *R. japonica*, showed no antifungal activity (MIC > 1000 µg mL⁻¹) (data not shown). Previous studies also reported the absence of antifungal activity of some species of *Baccharis* spp (e.g., aerial parts of *B. darwinii*, *B. microphylla*, *B. petiolata* and *B. santeliciis*; leaves of *B. dracunculifolia*, *B. aracetubaensis* and *B. organensis*, cladodes of *B. burchellii*) against *C. albicans*.^[19,20]

Interestingly, EtOH and EtOH-H₂O extracts of *R. japonica* leaves revealed the best antifungal activity, with MIC₅₀ of 250 µg mL⁻¹ and 15.6 µg mL⁻¹, respectively. The comparison of our results with those of Lee and Kim suggested that the leaves of *R. japonica* would be more efficient than the roots. Lee and Kim previously reported that the MIC of an EtOH extract of roots was 391 µg mL⁻¹.^[21]

All extracts were also tested at concentrations ranging between 62.5 and 500 µg mL⁻¹ against 24 h old biofilms of *C. albicans* ATCC 28367 to assess their effect on sessile yeasts (Figure 1). Finding a molecule capable of destroying an already formed mature biofilm is part of the long-term goal of providing a curative solution for biofilm-associated infections, as such a molecule is not yet available in the current therapeutic arsenal.

All extracts of *B. halimifolia*, except the hexane extract of *B. halimifolia* stems (Bh-S), were significantly active, inhibiting the preformed biofilms by more than 50% at concentrations ≥ 125 µg mL⁻¹ (p < 0.05). These results suggested the active compounds had various polarities, from polar to apolar. Indeed,

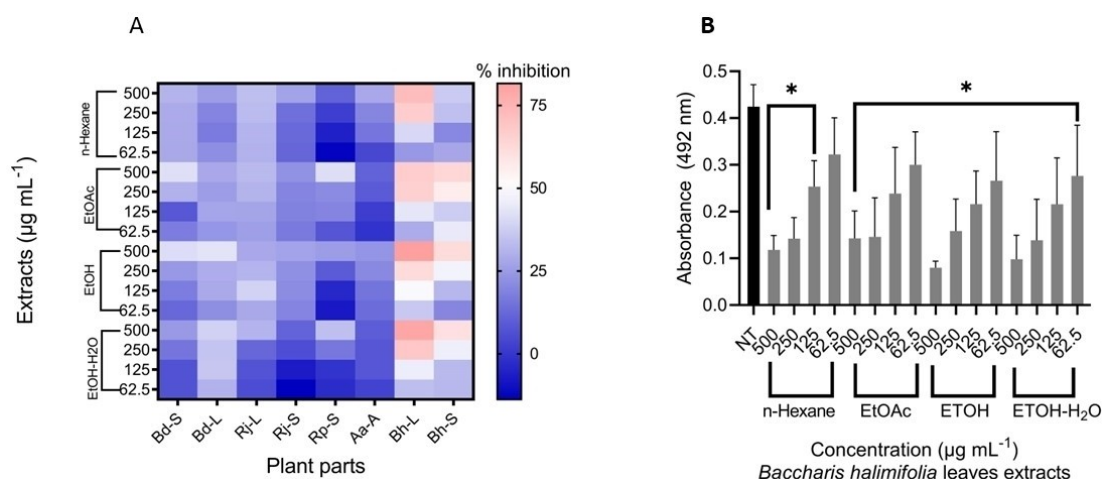


Figure 1. The activity of invasive plants extracts (A) and mean of absorbance values of the extracts of *Baccharis halimifolia* leaves (B) against *Candida albicans* ATCC 28367 24 h-biofilm. (A) The colored bar indicates the growth inhibition percentages. Bd-S (*Buddleja davidii* stems); Bd-L (*Buddleja davidii* leaves); Rj-L (*Reynoutria japonica* leaves); Rj-S (*Reynoutria japonica* stems); Rp-S (*Robinia pseudoacacia* stems); Aa-A (*Ambrosia artemisiifolia* aerial parts); Bh-L (*Baccharis halimifolia* leaves); Bh-S (*Baccharis halimifolia* stems). Two independent experiments were conducted (quadruplicate). (B) Results were compared to non-treated group (NT) and analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test (* $p < 0.05$).

it is known that polar compounds, as phenolic compounds, or apolar compounds, as terpenoids compounds, can inhibit biofilms, especially those formed by *C. albicans*.^[22]

The best activity was associated with *B. halimifolia* leaves (Bh-L) extracts, especially EtOH and EtOH-H₂O extracts which inhibited mature biofilms of *C. albicans* by more than 75% at the highest tested concentration (500 $\mu\text{g mL}^{-1}$). In contrast, extracts prepared from the six other parts of plants (Bd-S; Bd-L; Fj-L; Fj-S; Rp-S, Aa-A) never succeeded in inhibiting preformed biofilms by more than 50%, while some even tended to promote biofilm growth (Fj-S EtOH-H₂O, Rp-S hexane, and EtOH, Aa-A AcOEt at low concentrations).

As the largest genus in the *Asteraceae* family, *Baccharis* comprises more than a hundred species found natively all over the North and South American continents in which some *Baccharis* spp. were used in the traditional medicine such as for the treatment of diabetes, digestive, liver disease, respiratory disease, pain, and fever.^[21,22] The activity of extracts of some native *Baccharis* species (EtOH-H₂O extracts of *B. dracunculifolia* and *B. trimera* tincture, in agreement with our EtOH and EtOH-H₂O extracts) has already been shown against certain bacterial species (*Streptococcus mutans*, *Pseudomonas aeruginosa*, *Staphylococcus pseudintermedius*, *Staphylococcus aureus*).^[23–25] However, to our knowledge, we report for the first time the antibiofilm activity of *Baccharis* spp. against fungi by demonstrating its activity against *Candida* spp. biofilms.

Antifungal and antibiofilm activity of the Essential oil obtained from the leaves of *B. halimifolia*

B. halimifolia leaves (Bh-L) were extracted by hydrodistillation to obtain the essential oil (EO) (yield: 0.12%) as they showed the highest antibiofilm activity and were known for their high EO content.^[26] The antifungal activity and the activity against a

mature biofilm of *C. albicans* of EO were thus evaluated. Unfortunately, Bh-L EO displayed a weak antifungal activity (MIC = 5 mg mL^{-1}) (data not shown). This result was in agreement with those reported for other species of *Baccharis* (*B. trimera* and *B. oreophila*) highlighting an inhibition of the growth of *Candida* strains only when the EO was used at the highest concentrations (IC₅₀ = 5289.15 $\mu\text{g mL}^{-1}$ and MIC > 2500 $\mu\text{g mL}^{-1}$ against *C. albicans*, respectively).^[27,28]

Interestingly, our results showed that Bh-L EO inhibited 24 h-old-biofilm by 80% at 1000 $\mu\text{g mL}^{-1}$ ($p < 0.05$) and by 50% (IC₅₀) at 74.71 $\mu\text{g mL}^{-1}$, with 95%CI ranging from 59.52 $\mu\text{g mL}^{-1}$ to 93.55 $\mu\text{g mL}^{-1}$ (IC₅₀ determined using non-linear regression). Moreover *B. halimifolia* EO was able to significantly inhibit biofilms at low concentrations, as low as 3.91 $\mu\text{g mL}^{-1}$ ($p < 0.05$) (Figure 2A).

Literature data also reported the activity of some EO extracted from native *Baccharis* species against bacterial biofilms. For example, *B. dracunculifolia* EO was shown to disrupt *S. mutans* biofilm and the dental biofilm (in a mouth-

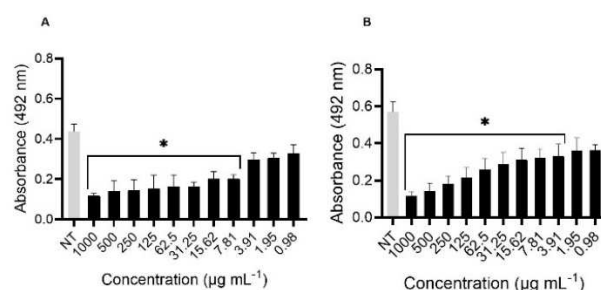


Figure 2. Antibiofilm activity of essential oil (EO) of *B. halimifolia* leaves on (A) 24 h-biofilm or (B) 3 h-maturation phase of *C. albicans* ATCC 28367. Two independent experiments were conducted (quadruplicate). Results were compared to non-treated group (NT) and analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test (* $p < 0.05$).

wash formulation), or *Baccharis psiadioides* EO inhibited the biofilm formation of *S. epidermidis* and *Enterococcus faecalis*.^[25,29,30] Thus, given the promising antibiofilm activity of Bh-L EO compared to the 32 tested extracts, Bh-L EO was selected for further investigation. To our knowledge, our study demonstrate for the first time the antibiofilm properties of *B. halimifolia* EO against *Candida albicans*.

Characterization of the antibiofilm activity of Bh-L EO

Activity of Bh-L EO against the biofilm maturation phase of *C. albicans*

To examine a potential preventive effect, an antimaturation test was conducted to gauge the impact of Bh-L EO on the initial phases of biofilm formation. *C. albicans* ATCC 28367 yeasts were treated for 24 h with EO once adhered, to specifically target the maturation phase. Bh-L EO used at concentrations down to $7.81 \mu\text{g mL}^{-1}$ induced a significant inhibition compared to the non-treated condition ($p < 0.05$) (Figure 2B). We observed that the Bh-L EO used at $1000 \mu\text{g mL}^{-1}$ inhibited biofilm maturation by 73.25% ($p < 0.05$), and IC_{50} of Bh-L EO was $4 \mu\text{g mL}^{-1}$, with 95%CI ranging from $2.76 \mu\text{g mL}^{-1}$ to $5.89 \mu\text{g mL}^{-1}$ (IC_{50} determined using non-linear regression). The EO could therefore be useful not only to eradicate a preformed biofilm but also to prevent its maturation and thus, delay the dispersion step which is highly responsible for the infection.

Activity of Bh-L EO against clinical strains of *C. albicans*

Clinical isolates and laboratory reference strains of *C. albicans* form biofilms at different rates.^[31] Additionally, our experience showed that clinical isolates were usually less susceptible to drugs and more difficult to combat than collection strains.^[32,33] For these reasons, it was essential to include clinical isolates to confirm the anti-*C. albicans* activity of Bh-L EO. The lowest IC_{50} values determined for antimaturation and antibiofilm activities were obtained for the clinical isolate CIS1 ($54.74 \mu\text{g mL}^{-1}$ and $59.85 \mu\text{g mL}^{-1}$, respectively, $p < 0.05$) (Table 1).

The antibiofilm value was comparable to that obtained using *C. albicans* ATCC 28367 ($59.85 \mu\text{g mL}^{-1}$ vs. $74.71 \mu\text{g mL}^{-1}$). It is contrary to the antimaturation value which was higher

($54.74 \mu\text{g mL}^{-1}$ vs. $4 \mu\text{g mL}^{-1}$) showing a decrease of activity. The three other clinical isolates showed IC_{50} values higher than $210 \mu\text{g mL}^{-1}$. These results suggested a strain dependence and a partial loss of activity against clinical strains confirming the reduced susceptibility of clinical isolates to the treatments.

Antimaturation and antibiofilm tests against other *Candida* species

To find out whether the antimaturation and antibiofilm activity were species-dependent, three *Candida* species, which also involved in human pathologies, were selected: *C. glabrata*, *C. krusei*, and *C. parapsilosis*. Bh-L EO inhibited 24 h-old biofilm and maturation phase of *C. krusei* ($p < 0.05$) when using concentrations of at least $31.25 \mu\text{g mL}^{-1}$ and the highest tested concentration ($1000 \mu\text{g mL}^{-1}$) strongly inhibited both biofilms preformed (40.6%) and in formation (46.7%) (Figure 3). Regarding *C. glabrata*, Bh-L EO at $1000 \mu\text{g mL}^{-1}$ strongly inhibited biofilm maturation phase (60%, $p < 0.05$) and a lower activity was maintained using concentrations of at least $7.81 \mu\text{g mL}^{-1}$, with inhibition percentages ranging from 15% at $7.81 \mu\text{g mL}^{-1}$ to 25% at $500 \mu\text{g mL}^{-1}$, $p < 0.05$). It was found that Bh-L EO did not reach to reduce 24 h-old-biofilms of *C. glabrata*, regardless of the concentrations. *C. parapsilosis* was the least sensitive to the Bh-L EO on both targeted phases ($\leq 21\%$ of inhibition). To conclude, the inhibition activity was not specific to *C. albicans*. However, this species remains the most sensitive to the effects of Bh-L EO. This difference in sensitivity between species could be explained by differences in biofilm formation and composition. *C. parapsilosis* biofilms, for example, are thinner and less structured than *C. albicans*, and composed entirely of aggregated blastospores.^[34] Furthermore, the composition of the exopolymeric matrix varies by species. Its total carbohydrate and protein content have been shown to be higher in non-*Candida albicans* species (including *C. krusei*, *C. parapsilosis*, and *C. glabrata*) than in *C. albicans*.^[34,35]

Ability of Bh-L EO to inhibit a bi-species biofilm: *C. albicans* and *S. aureus*

In nature, biofilms are rarely single species, and it is known that the co-presence of several species can modulate the response of each to a drug.^[36,37] To confirm Bh-L EO's interest in eradicating sessile *C. albicans* yeasts, it was essential to investigate the effect of bacteria presence on its activity using interkingdom biofilms. The persistence of the inhibitory activity observed with *C. albicans* ATCC 28367 was thus evaluated in the presence of a bacterial species sharing the same niches: *S. aureus*, and anti-biofilm tests were conducted by crystal violet staining.

Single-species *S. aureus* ATCC 29213 biofilm (Sa), single-species *C. albicans* ATCC 28367 biofilm (Ca), and dual-species (*S. aureus* ATCC 29213 + *C. albicans* ATCC 28367) biofilms (Sa + Ca) were prepared. The crystal violet approach allowed to evaluate the effect of Bh-L EO on the biomass quantity. The

Table 1. Antibiofilm activity of essential oil of *B. halimifolia* leaves on 3 h-maturation phase and 24 h biofilm of four clinical isolates of *C. albicans*. Values are expressed as IC_{50} (with confidence interval 95% – CI 95%) determined by non-linear regression. The test was performed in quadruplicate in two independent experiments.

Candida strains	3 h-maturation phase ($\mu\text{g mL}^{-1}$)	24 h-biofilm ($\mu\text{g mL}^{-1}$)
	IC_{50} (95%CI)	IC_{50} (95%CI)
CIS1	54.74 (38.86–76.9)	59.85 (42.60–83.77)
CIS2	> 1000 (> 1000)	338.7 (311.7–374.1)
CIS3	210.6 (176.9–249.7)	306.0 (284.8–327.4)
CIS4	586 (515.5–757.7)	587.2 (416.6–668.1)

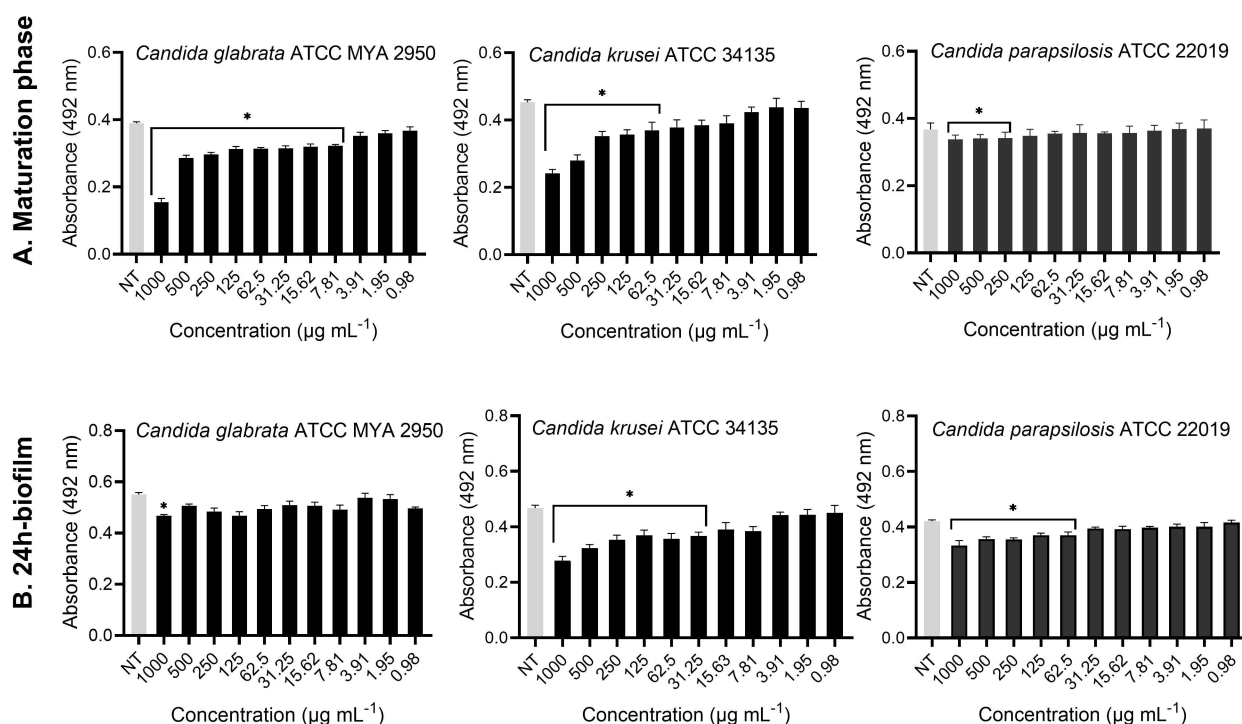


Figure 3. Antibiofilm activity of essential oil (EO) of *B. halimifolia* leaves on A) 3 h-maturation phase and B) 24 h-biofilm of *C. glabrata* ATCC MYA 2950, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 34135. Two independent experiments were conducted (quadruplicate). Results were compared to non-treated group (NT) and analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test (* $p < 0.05$).

obtained results confirmed the activity of Bh-L EO on *C. albicans* single-species biofilms (66% of inhibition at $7.81 \mu\text{g mL}^{-1}$, Figure 4A) previously highlighted by the XTT approach, which measured the metabolic activity (50% of inhibition at $74.71 \mu\text{g mL}^{-1}$, Figure 2A). However, no inhibition was observed against either *S. aureus* single-species biofilms or *S. aureus*-*C. albicans* bi-species biofilms, suggesting the loss of the inhibitory activity in the presence of *S. aureus*. This result is disappointing but can be explained by the interaction and

communication means used by these pathogens. For example, it has been shown that *S. aureus* can up-regulate *C. albicans* ergosterol biosynthesis genes and drug-resistance transporter (drug-efflux pumps) genes.^[38]

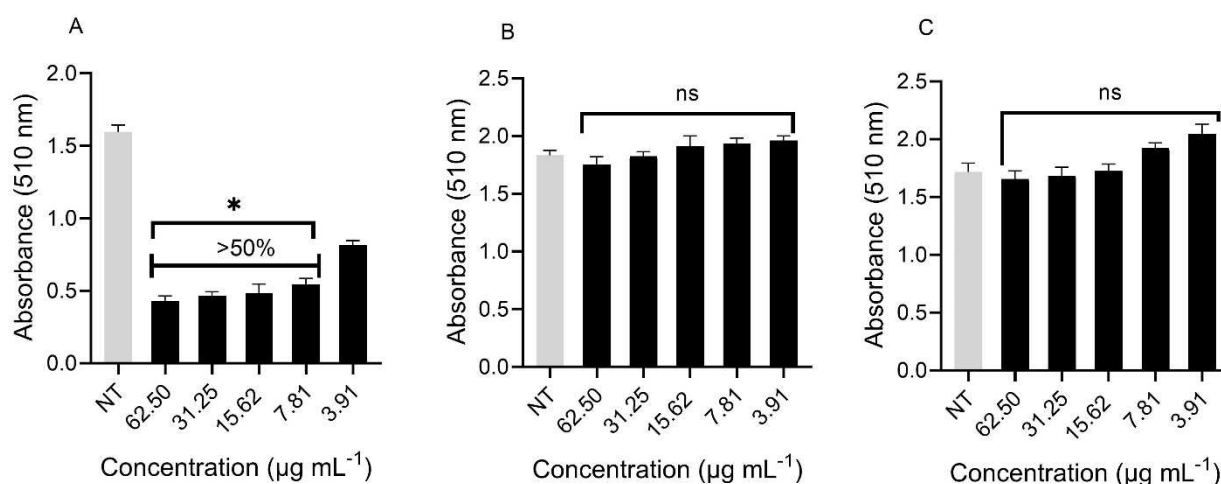


Figure 4. Effect of EO of *B. halimifolia* leaves on (A) monospecies 24 h *C. albicans* ATCC 28367 biofilm, (B) dual species *S. aureus* ATCC 29213 + *C. albicans* ATCC 28367, and (C) monospecies *S. aureus* ATCC 29213. The biomass was quantified using crystal violet staining. Three independent experiments were performed (triplicate). ns = not significant. * $P < 0.05$ calculated by Dunn's multiple comparisons test.

Chemical composition of essential oil obtained from the leaves of *B. halimifolia*

According to the results of GC/MS analysis, 48 components were detected, accounting for 96.62% of the total EO composition (Table 2). Oxygenated sesquiterpenes were present in the majority, accounting for 62.02%. This observation was in line with that done for other species of *Baccharis*, such as *B. latifolia* (oxygenated sesquiterpenes: 69.8%), *B. uncinella* (68.42%) or *B. dracunculifolia* (60.8%).^[39–41] Nevertheless, the major compounds were different: γ -Curcumene (12.2%) and Spathulenol (32.93% and 27.43%) were the main compounds of *B. latifolia*, *B. uncinella*, and *B. dracunculifolia*, respectively. In our study, the most abundant compound was β -caryophyllene oxide (37.54%) followed by humulene epoxide II (8.12%). A previous study of the composition of EO from *B. halimifolia* leaves identified 64 compounds and showed that β -pinene was the major component (11.9%) – while in this present study, it was only 1.44% – followed by caryophyllene oxide (7%) and α -humulene epoxide II (6.2%).^[42] Those differences were not surprising, as the chemical constituents of the EO depend on the climatic and geographical conditions, plant age, and the distillation technique used.^[43]

Some of the forty-eight potentially identified compounds were commercially available and were thus selected for investigation to examine their involvement in the activity of the Bh-L EO.

Antifungal and antibiofilm activities of selected compounds

Four commercial compounds – aromadendrene oxide-(2); β -caryophyllene; β -caryophyllene oxide; and ($\pm$)- β -pinene (three major sesquiterpenes and one monoterpene) were examined to evaluate their antifungal and antibiofilm activities (Figure S1). As for Bh-L EO, a weak antifungal activity was observed with the four pure compounds against *C. albicans* but also against other species of *Candida* (*C. krusei*, *C. parapsilosis*, and *C. glabrata*) ($\text{MIC}_{50} \geq 10^{-3} \text{ mol l}^{-1}$ ($\geq 220 \mu\text{g mL}^{-1}$)) (Table 3). Only the activity against *C. krusei* of aromadendrene oxide-2 was closed to that of fluconazole ($220 \mu\text{g mL}^{-1}$ vs. $101 \mu\text{g mL}^{-1}$). A weak activity was also reported in the literature for (+)- β -pinene and (–)- β -pinene against *C. albicans* ($\text{MIC} \geq 187 \mu\text{g mL}^{-1}$) and β -caryophyllene against *C. albicans* and *C. parapsilosis* ($\text{MIC} > 2.5 \text{ mg mL}^{-1}$).^[51,52] Some authors reported a MIC of β -caryophyllene oxide lower than $60 \mu\text{g mL}^{-1}$ on *C. albicans*, which is significantly lower than that we found (MIC : $440 \mu\text{g mL}^{-1}$).^[53]

Table 2. Chemical composition of essential oil of *Baccharis halimifolia* leaves.

Compounds	Tr[min]	KI exp	%	Compounds	Tr[min]	KI exp	%
Monoterpene hydrocarbons				Oxygenated sesquiterpenes			
α -Pinene	5.54	933	0.07	β -Caryophyllene oxide	23.43	1639	37.54
β -Pinene	6.84	982	1.44	Humulene epoxide II	25.14	1592	8.12
β -Myrcene	7.21	996	0.04	5,5-Dimethyl-4-(3-methyl-1,3-butadienyl)-1-oxaspiro[2.5]octane	25.29	1673	1.09
Cyclohexanol	8.53	1042	0.44	Cubenol	25.43	1679	2.43
7,7-Dimethylbicyclo[2.2.1]heptan-2-yl acetate	43.60	1247	0.65	Aromadendrene oxide	25.56	1685	1.56
Dolichodial	13.20	1256	0.27	4,4-Dimethyltetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol	25.70	1691	3.41
Oxygenated monoterpenes				β -Eudesmol	26.07	1707	0.41
Linalool	10.69	1116	0.73	Widdrol	26.29	1716	0.62
<i>trans</i> -Pinocarveol	11.98	1160	0.69	Isoaromadendrene epoxide	26.30	1717	0.92
Pinocarvone	12.89	1192	0.62	Aromadendrene oxide-(2)	26.40	1721	2.47
Myrtenal	13.98	1230	0.85	9-Isopropyl-1-methyl-2-methylene-5-oxatricyclo[5.4.0.0(3,8)]undecane	26.92	1744	1.63
α -Terpineol	13.86	1226	0.25	Ledene oxide-(II)	27.24	1748	1.09
Myrtenol	13.69	1220	0.38	Murolan-3,9(11)-diene-10-peroxy	29.15	1839	0.52
<i>trans</i> -Pinocarvyl acetate	16.48	1320	0.68	6,10,14-Trimethyl-2-pentadecanone	29.76	1864	0.21
Sesquiterpene hydrocarbons				Others			
α -Cubebene	17.62	1362	0.43	Methyl 3,3-dimethylbicyclo[2.2.1]heptane-2-carboxylate	15.25	1275	4.91
Ylangene	18.52	1396	2.63	Ethyl 3,7-dimethylocta-2,6-dienoate	16.19	1309	0.44
β -Elemene	18.97	1413	1.83	Acetic acid, (1,2,3,4,5,6,7,8-octahydro-3,8,8-trimethylnaphth-2-yl) methyl ester	27.46	1768	0.21
Isolongifolene,9,10-dehydro-	19.45	1432	1.30	3,7,11,15-Tetramethyl-2-hexadecan-1-ol	29.26	1843	0.39
β -Caryophyllene	19.72	1443	3.23	6-(1,3-Dimethyl-buta-1,3-dienyl)-1,5,5-trimethyl-7-oxa-bicyclo[4.1.0]hept-2-ene	29.55	1855	0.14
Humulene	20.68	1480	1.24	Methyl arachidonate	30.29	1886	0.05
β -Cadinene	21.08	1496	0.78	(8S)-1-Methyl-4-isopropyl-7,8-dihydroxyspiro[tricyclo[4.4.0.0(5,9)]decane-10,2'-oxirane]	30.95	1917	0.22
γ -Murolene	21.24	1502	1.03	Total identified			96.62
Germacrene D	21.44	1510	0.50	Monoterpene hydrocarbons			2.91
Eudesma-4(14),7(11)-diene	21.68	1520	1.07	Oxygenated monoterpenes			4.20
α -Murolene	21.88	1529	0.97	Sesquiterpene hydrocarbons			21.13
δ -Cadinene	22.33	1547	3.32	Oxygenated sesquiterpenes			62.02
<i>cis</i> -Calamene	22.58	1558	0.49	Others			6.36
α -Calacorene	23.18	1582	2.31				

Tr [min] = Retention time in min; KI exp = Kovats index experiment; % = the percentage of compound content from the total oil composition.

Table 3. Antifungal, antimaturation, and antibiofilm activities of aromadendrene oxide-(2), β -caryophyllene oxide, ($\pm$)- β -pinene, and β -caryophyllene on *Candida* species. MIC on planktonic cells was determined by visual end-points (CLSI M27-A3). The antimaturation and antibiofilm activities were expressed as 50% inhibitory concentration (IC_{50}), calculated by using non-linear regression with $p < 0.05$ (GraphPad Prism). *NT = Not Tested.

	Aromadendrene oxide-2	β -Caryophyllene oxide	($\pm$)- β -Pinene	β -Caryophyllene	Fluconazole
MIC on planktonic cells ($mol\ l^{-1}$)					
<i>C. albicans</i> ATCC 28367	10^{-3}	2×10^{-3}	3×10^{-3}	$> 2 \times 10^{-3}$	0.02×10^{-3}
<i>C. glabrata</i> ATCC MYA 2950	10^{-3}	2×10^{-3}	1.5×10^{-3}	$> 2 \times 10^{-3}$	0.04×10^{-3}
<i>C. krusei</i> ATCC 34135	10^{-3}	$> 2 \times 10^{-3}$	$> 3 \times 10^{-3}$	$> 2 \times 10^{-3}$	0.33×10^{-3}
<i>C. parapsilosis</i> ATCC 22019	$> 2 \times 10^{-3}$	2×10^{-3}	$> 3 \times 10^{-3}$	$> 2 \times 10^{-3}$	0.01×10^{-3}
IC_{50} on 3 h-maturation phase ($mol\ l^{-1}$)					
<i>Clinical Candida albicans</i>					
S1	0.01×10^{-3}	0.32×10^{-3}	4.88×10^{-3}	$> 4 \times 10^{-3}$	NT
S2	2.27×10^{-3}	$> 4 \times 10^{-3}$	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
S3	1.27×10^{-3}	0.046×10^{-3}	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
S4	1.24×10^{-3}	$> 4 \times 10^{-3}$	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
<i>Candida non-albicans</i>					
<i>C. glabrata</i> ATCC MYA 2950	0.59×10^{-3}	3.91×10^{-3}	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
<i>C. krusei</i> ATCC 34135	2.26×10^{-3}	$> 4 \times 10^{-3}$	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
<i>C. parapsilosis</i> ATCC 22019	$> 4 \times 10^{-3}$	$> 4 \times 10^{-3}$	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
IC_{50} on 24 h-biofilm ($mol\ l^{-1}$)					
<i>Clinical Candida albicans</i>					
S1	1.20×10^{-3}	1.23×10^{-3}	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
S2	1.56×10^{-3}	0.20×10^{-3}	1.46×10^{-3}	$> 4 \times 10^{-3}$	NT
S3	1.88×10^{-3}	$> 4 \times 10^{-3}$	9.5×10^{-4}	$> 4 \times 10^{-3}$	NT
S4	3.63×10^{-3}	$> 4 \times 10^{-3}$	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
<i>Candida non-albicans</i>					
<i>C. glabrata</i> ATCC MYA 2950	1.19×10^{-3}	$> 4 \times 10^{-3}$	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
<i>C. krusei</i> ATCC 34135	2.47×10^{-3}	$> 4 \times 10^{-3}$	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT
<i>C. parapsilosis</i> ATCC 22019	$> 4 \times 10^{-3}$	$> 4 \times 10^{-3}$	$> 6 \times 10^{-3}$	$> 4 \times 10^{-3}$	NT

However, this comparison is questionable because the authors used an agar dilution method instead of a microdilution approach in our case.

Interestingly some compounds would display antifungal effects on other fungi such as dermatophytes: *Trichophyton rubrum* for β -caryophyllene and caryophyllene oxide and *Trichophyton mentagrophytes* sp. for β -caryophyllene oxide.^[52,54]

Regarding activity against biofilms, the three compounds – aromadendrene oxide-(2), β -caryophyllene oxide, and ($\pm$)- β -pinene – showed significant activities in a dose-dependent manner against *C. albicans* ATCC 28367; IC_{50} evaluated for antimaturation tests were $0.18 \times 10^{-3} mol\ l^{-1}$ ($39.5\ \mu g\ mL^{-1}$), $0.09 \times 10^{-3} mol\ l^{-1}$ ($19.7\ \mu g\ mL^{-1}$), and $0.34 \times 10^{-3} mol\ l^{-1}$ ($46.33\ \mu g\ mL^{-1}$). While IC_{50} obtained for antibiofilm tests were $0.26 \times 10^{-3} mol\ l^{-1}$ ($57.3\ \mu g\ mL^{-1}$), $0.038 \times 10^{-3} mol\ l^{-1}$ ($8.3\ \mu g\ mL^{-1}$), and $0.63 \times 10^{-3} mol\ l^{-1}$ ($85.8\ \mu g\ mL^{-1}$), respectively (Figure 5).

Thus, β -caryophyllene oxide showed the highest activity. β -Caryophyllene displayed very weak antimaturation and antibiofilm activity (9% and 12.8% of inhibition, respectively at the highest tested concentration: $4 \times 10^{-3} mol\ l^{-1}$ ($817.4\ \mu g\ mL^{-1}$)) (data not shown). This weak activity suggested that the presence of the epoxy group on the caryophyllene structure would be involved in the activity (Figure S1).

The most essential aspect of epoxides is their electrophilic and lipophilic nature, allowing these compounds to interact with the membrane components and to permeate through cell membranes.^[44] β -Caryophyllene oxide can modify the packing of the phospholipids and the stability of the bilayers of the membrane.^[44] So far, no study evaluated the effect of β -caryophyllene oxide against *Candida* spp. biofilm, especially

focusing on the extracellular matrix. However, such relationships deserve to be investigated. Indeed, the matrix contains a variety of lipids, including phospholipids (phosphatidylcholine and phosphatidylethanolamine), sphingolipids, and eicosanoids which are crucial to the *C. albicans* pathogenesis. In addition, the disruption of those phospholipids biosynthesis causes a virulence of *C. albicans*.^[45,46]

To our knowledge, our study shows for the first time the antimaturation and antibiofilm activities of aromadendrene oxide-(2), as well as β -caryophyllene oxide against *Candida* species. Regarding β -pinene, da Silva and collaborators and Raut and collaborators already reported some antibiofilm activity against *C. albicans*. It is da Silva and collaborators who reported that (+)- β -pinene reduced biofilm formation by 54% at MIC $187\ \mu g\ mL^{-1}$. Furthermore, Raut and collaborators observed that β -pinene (the type of enantiomer is not specified) inhibited the biofilm in formation and once mature with MIC $\geq 4\ mg\ mL^{-1}$.^[47,48] Their data were thus different from ours (IC_{50} antimaturation: $46.33\ \mu g\ mL^{-1}$ and IC_{50} antibiofilm $85.8\ \mu g\ mL^{-1}$). These differences can be explained by the use of various enantiomers of β -pinene and by the variety of methods (culture medium, cell counts method, starting inoculum, etc.) due to an absence of international standardized protocol for antibiofilm tests contrary to antifungal assay (EUCAST/CLSI). Thus, the inhibitory activity of Bh-L EO against *C. albicans* biofilm could be explained by the presence in its chemical composition of aromadendrene oxide-(2), β -pinene and above all β -caryophyllene oxide which is its major component. The fact that Bh-L EO appeared more active against the maturation than these three compounds used separately (IC_{50} : $4\ \mu g\ mL^{-1}$ versus $[19.7\text{--}49.05\ \mu g\ mL^{-1}]$), highlighted a possible synergy between the EO

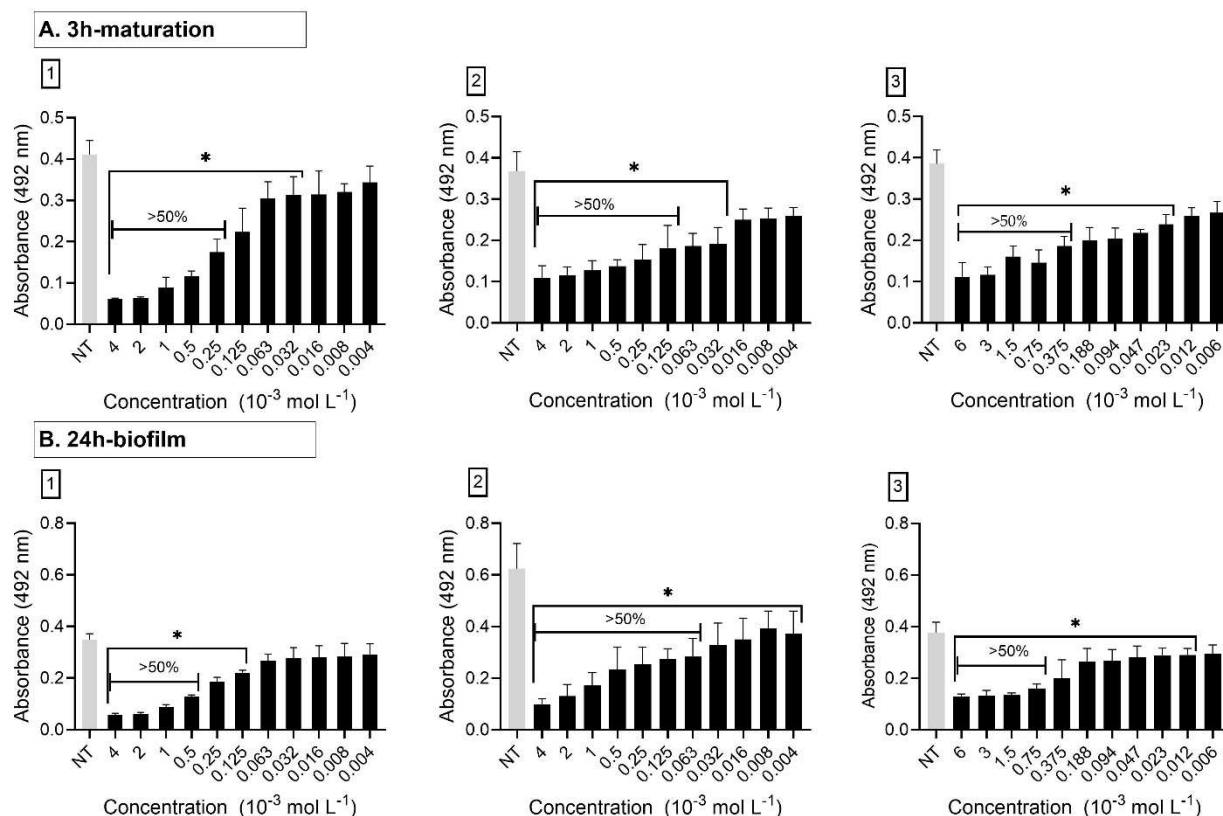


Figure 5. Antibiofilm activity of aromadendrene oxide-(2) (1), β -caryophyllene oxide (2), and $(\pm)$ - β -pinene (3) on A) 3 h-maturation phase and B) 24 h-biofilm of *C. albicans* ATCC 28367. Two independent experiments were conducted (quadruplicate). Results were compared to non-treated group (NT) and analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test (* $p < 0.05$).

compounds. To confirm their interest, the four compounds were tested against the four clinical isolates of *C. albicans* (table 3). Globally, in line with what was observed with Bh-L EO, a loss of activity was obtained using the clinical isolates and a certain strain dependence can be noted for these compounds. Only aromadendrene oxide-(2) retained significant antimaturation and antibiofilm activities on all tested strains. These activities were often obtained using high concentrations ($[1.20 \times 10^{-3} - 3.63 \times 10^{-3} \text{ mol L}^{-1}]$) for clinical isolates versus $[0.18 \times 10^{-3} - 0.26 \times 10^{-3} \text{ mol L}^{-1}]$ in the case of the reference strain, except for S1 which was more susceptible (0.01×10^{-3} versus $0.18 \times 10^{-3} \text{ mol L}^{-1}$) in antimaturation test. For both tests, only two clinical strains were significantly inhibited by β -caryophyllene oxide and at concentrations higher than those needed in the case of the reference strain ($[0.04 \times 10^{-3} - 1.23 \times 10^{-3} \text{ mol L}^{-1}]$ versus $0.09 \times 10^{-3} - 0.038 \times 10^{-3} \text{ mol L}^{-1}$), except S3 which was more susceptible (antimaturation test: 0.04×10^{-3} versus $0.09 \times 10^{-3} \text{ mol L}^{-1}$). Finally, the clinical isolates S1, S2 and S3 were inhibited (antimaturation and antibiofilm tests, $p < 0.05$) by $(\pm)$ - β -pinene ($\text{IC}_{50} \leq 4.88 \times 10^{-3} \text{ mol L}^{-1}$).

The antimaturation and antibiofilm activities of the four compounds were also evaluated against the three other *Candida* species (Table 3). Only aromadendrene oxide (2) significantly inhibited *C. glabrata* and *C. krusei*, however at the highest concentrations than for *C. albicans* (IC_{50} :

$0.59 \times 10^{-3} - 2.47 \times 10^{-3} \text{ mol L}^{-1}$ versus $0.18 \times 10^{-3} - 0.26 \times 10^{-3} \text{ mol L}^{-1}$). Similar to Bh-L EO, *C. albicans* remained the most susceptible species. *C. parapsilosis* biofilms were not inhibited, whatever the tested compound, as previously observed for Bh-L EO. The three compounds β -caryophyllene, β -caryophyllene oxide, and $(\pm)$ - β -pinene were inactive against the *Candida* non-albicans tested species ($\text{IC}_{50} \geq 3.91 \times 10^{-3} \text{ mol L}^{-1}$). Thus, the presence of aromadendrene oxide (2) in the composition of EO could justify the antimaturation and/or antibiofilm activities observed with Bh-L EO against *C. krusei* and *C. glabrata*. And we can note that aromadendrene oxide (2) was more active than Bh-L EO against *C. krusei* and *C. glabrata* but always displayed moderate activity.

Cytotoxic activity

The cytotoxic activity of Bh-L EO and the three active compounds, i.e., aromadendrene oxide-(2), β -caryophyllene oxide, and $(\pm)$ - β -pinene were evaluated on the HeLa cell lines. Cells were treated by each sample at concentrations corresponding to $\text{IC}_{\text{biofilm}50}$ to 4-fold $\text{IC}_{\text{biofilm}50}$ values. Bh-L EO and compounds revealed different cytotoxic activities after a 24 h contact, as summarized in Table 4. β -Caryophyllene oxide showed the lowest cytotoxicity, whatever the tested concentrations (at most 4% cytotoxicity). It is in accordance with the

Table 4. Cytotoxic activity of Essential oil (EO) of *B. halimifolia* leaves and some pure compounds. Data are presented as mean $\pm$ SD of two independent experiments performed in triplicate. Non-linear regression (GraphPad Prism) was used to determine $IC_{\text{cytotox50}}$ values (95%CI) and values are expressed in $\mu\text{g mL}^{-1}$.

Samples	$IC_{\text{biofilm50}}$ (95%CI) ($\mu\text{g mL}^{-1}$)	$IC_{\text{cytotox50}}$ (95%CI) ($\mu\text{g mL}^{-1}$)	Cytotoxicity on HeLa cells (% $\pm$ SD)		
			$4IC_{\text{biofilm50}}$	$2IC_{\text{biofilm50}}$	$IC_{\text{biofilm50}}$
Essential oil of <i>B. halimifolia</i> leaves	74 (59.52 to 93.55)	249 (153.7–675.0)	54.34 ± 0.02	32.29 ± 0.04	29.27 ± 0.05
Aromadendrene oxide-(2)	57.3 (50.44–63.6)	75.33 (69.37–77.35)	93.36 ± 0.01	93.22 ± 0.01	0.70 ± 0.05
β -Caryophyllene oxide	8.3 (4.38–13.16)	42.80 (42.16–43.48)	4.34 ± 0.16	1.20 ± 0.08	-0.89 ± 0.17
($\pm$)- β -Pinene	85.8 (53.13–147.2)	157.3 (137.9–178.4)	84.47 ± 0.11	57.87 ± 0.48	14.59 ± 0.10

Note: $4IC_{\text{biofilm50}}$ = 4-fold 50% Biofilm Inhibitory Concentration; $2IC_{\text{biofilm50}}$ = 2-fold 50% Biofilm Inhibitory Concentration; $IC_{\text{biofilm50}}$ = at 50% Biofilm Inhibitory Concentration against 24 h-*Candida albicans* biofilm.

fact that β -caryophyllene oxide is used as flavour and fragrance and it is enclosed in the European list of flavourings substances, with the identification number of FL no. 16.043.^[49] A report of EFSA concluded that there no safety concern at the estimated level of intake.^[50] Aromadendrene oxide-(2) also exhibited low cytotoxicity but only at a concentration corresponding to the antibiofilm IC_{50} value (0.7% at $57.3 \mu\text{g mL}^{-1}$). In this sense, literature data reported that this compound did not exhibit toxicity against human mesenchymal stem cells up to $200 \mu\text{M}$ ($44 \mu\text{g mL}^{-1}$). However, literature data also suggested that cytotoxicity depends on the studied cells. Indeed, aromadendrene oxide-(2) induced also a cytotoxic effect on A431 human epidermoid cancer and on precancerous HaCaT cells, displaying IC_{50} values of $50 \mu\text{M}$ ($11.0 \mu\text{g mL}^{-1}$) and $76 \mu\text{M}$ ($16.7 \mu\text{g mL}^{-1}$), respectively.^[51] Bh-L EO showed the highest cytotoxicity at concentration corresponding to the antibiofilm IC_{50} value, with nearly 30%. Our research provides the first data on the cytotoxicity of *B. halimifolia* essential oil.

Conclusions

To the best of our knowledge, this is the first report on the activity of EO obtained from *B. halimifolia* leaves on biofilm maturation and on preformed biofilms of *Candida* spp. Our results suggested that this EO could have an interest in the search of prophylactic and curative strategies in order to handle some biofilm related infections. According to our results, the activity of Bh-L EO was probably due to the presence of aromadendrene oxide-(2), ($\pm$)- β -pinene and above all of its major compound: β -caryophyllene oxide. Unfortunately, Bh-L EO showed cytotoxicity at the concentration responsible for the anti-biofilm activity. Promisingly, its main component, β -Caryophyllene oxide, displayed activity on *C. albicans* both on the early stage of the biofilm formation (maturation phase) and on the biofilm already formed, without toxicity. However, some strain-dependency was observed with this component. Based on the structure-activity relationship analyses, the presence of an epoxide functional group could be responsible for the activity of β -Caryophyllene oxide. It should also be noted that

aromadendrene oxide-(2) which is part of Bh-L EO composition demonstrated antibiofilm activity regardless of the studied phase; interestingly it displayed no strain-dependence but unfortunately was more cytotoxic at 2-fold $IC_{\text{biofilm50}}$. Further investigations are needed such as performing a pharmacomodulation study to reduce the toxicity and the strain dependency of these promising compounds. Elucidating their mechanisms of action and the persistence of their effectiveness should also be planned.

Experimental Section

Plant material and extraction

Two invasive plants, *Buddleja davidii* Franch and *Ambrosia artemisiifolia* L., were collected in August and September 2017 in Morre and Saint-Martin-de-Lerm (France), respectively (GPS positioning: *B. davidii* 47.225708, 6.072705, *A. artemisiifolia* 44.639556, -0.036361). Two other invasive alien plants, i.e., *Reynoutria japonica* Houtt. and *Robinia pseudoacacia* L. were collected in November 2019 near and in Poitiers (France) (GPS positioning: *R. japonica* 46.570750, 0.314960 and *R. pseudoacacia* 46.569166, 0.304138). At last, *Baccharis halimifolia* L. was collected in August 2020 also near Poitiers (France) (GPS positioning: 46.345299, -1.379955). Plants were harvested in accordance with national regulations. Botanical identification was performed by the Direction of the green spaces of the city of Poitiers, Dr. Marion Girardot (University of Poitiers) and Dr. Marion Millot (University of Limoges). Plants were air-dried for one week. A voucher specimen of each plant was deposited at the Herbarium of the School of Pharmacy at the University of Poitiers or at the University of Limoges (France) (registration numbers: BD 082017; AA 092017; RJ 112019; RP 112019; BH 082020).

Different parts of these plants (*R. japonica* leaves and stems, *A. artemisiifolia* aerial parts, *B. davidii* leaves and stems, *R. pseudoacacia* stems and *B. halimifolia* leaves and stems) were powdered using grinder and 5 g of powder were extracted by maceration assisted by sonication for 1 h at room temperature with a solvent: hexane or ethyl acetate or ethanol or ethanol-water (1:1). After centrifugation at 6000 rpm for 5 min (Beckman Coulter, Indianapolis, US), the supernatant of each extract was collected in a round bottom flask, evaporated under low pressure at 40°C (rotavapor Buchi, Flawil, Switzerland) and then kept at 4°C in airtight container until further analysis. For the extraction of *B. halimifolia* essential oil

(Bh-L EO), 300 g of leaves (no grinding) were subjected to hydro-distillation by using a Clevenger apparatus for 3.5 h. The recovered EO sample was stored at 4 °C.

Gas chromatography-mass spectrometry (GC/MS) analysis

The chemical composition of Bh-L EO was studied by GC/MS according to the method described by Chaftar and collaborators with some adaptations.^[52] This study was performed using an HP 6890 series chromatograph coupled to an HP 5973 mass selective detector (Agilent technologies, Santa Clara, USA) and equipped with a RESTEK RT 1614 column (30 m, 0.25 mm, 0.1 µm) (Restek, Lisses, France). The oven temperature programmed isothermal at 50 ° for 1 min, rising from 50 to 180 ° at 5 ° per minute, isothermal at 180 ° for 2 min, rising from 180 to 250 ° at 10 ° per minute, and finally isothermal at 250 ° for 2 min. Bh-L EO and a n-alkanes C₇-C₃₀ mixture (Merck, Darmstadt, Germany) were diluted (10⁻³ and 10⁻² v/v in hexane, respectively) before automatic injection. Identification of compounds was performed by computer matching against commercial library mass spectra (NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library database version 2.0 d build Dec, 2 2005) and determination of Kovats retention index. Quantitative analysis of Bh-L EO components (expressed as area percentages) was carried out by measuring the ratio of individual area and total area.

Biological tests

Strains

Reference strains of four *Candida* species and *Staphylococcus aureus* were used: *C. albicans* ATCC 28367, *C. glabrata* ATCC MYA 2950, *C. krusei* ATCC 34135, *C. parapsilosis* ATCC 22019 and *S. aureus* ATCC 29213, were purchased from the American Type Culture Collection (ATCC) (Manassas, USA). Four clinical isolates of *C. albicans* (CIS1, CIS2, CIS3, and CIS4) recovered from venous catheter of patients (provided by the laboratory of parasitology and medical mycology of the University hospital of Poitiers, France) were also used in this study.

Samples preparation

Stock solutions of extracts, Bh-L EO and commercial compounds (aromadendrene oxide-(2), β-caryophyllene oxide, (±)-β-pinene and β-caryophyllene) (Merck, Darmstadt, Germany) were prepared in DMSO at 50 mg mL⁻¹, 4% (v/v) and 20 mg mL⁻¹, respectively. The final DMSO concentration for all samples was always kept below 2% in each well of microplates during the biological tests.

Antifungal activity

Microdilution assay was adapted from Clinical and Laboratory Standards Institute (CLSI)-M27-A3 guidelines, with slight modifications, to determine the minimum inhibitory concentrations (MICs) of samples.^[53] Each *Candida* species was streaked on a Sabouraud glucose agar plate with chloramphenicol (0.05 g L⁻¹) (SGC) (Merck, Darmstadt, Germany) and incubated for 24 h at 37 °C. Several colonies from these cultures were picked up and suspended in RPMI 1640 (Merck, Darmstadt, Germany)-buffered with 0.165 mol L⁻¹ of 3-(N-morpholino) propanesulfonic acid (MOPS) (Merck, Darmstadt, Germany) medium. The concentration was calculated by direct counting with a Fast-Read 102® counting chamber (Biosigma, Venice, Italy) and adjusted with RPMI-MOPS medium to obtain a working suspension of approximately 5 × 10³ cells per mL. The

100 µL of working yeast suspension were then added to wells of 96-well microtiter plates containing 100 µL of a serial two-fold dilution in RPMI 1640-MOPS medium of extracts, Bh-L EO or commercial compounds stock solutions. The final concentrations obtained ranged between 1000 and 0.49 µg mL⁻¹ for extracts, 10 and 0.0049 mg mL⁻¹ for Bh-L EO, and 4 × 10⁻³ and 0.0039 × 10⁻³ mol L⁻¹ for commercial compounds, except for (±)-β-pinene which was prepared at concentrations ranging between 6 × 10⁻³ and 0.006 × 10⁻³ mol L⁻¹. Some wells were preserved for controls: non-treated yeasts (negative control), yeasts treated by fluconazole (positive control), and yeasts treated by DMSO 2% (DMSO control). According to CLSI -M27 A3 guidelines, MICs values were visually determined after incubation for 24 and 48 h at 37 °C by a prominent decrease in turbidity. Wells were scored for growth compared to that of the negative control wells (for example: a score of 2 = approximately 50% of inhibition).^[53] Each condition was performed in triplicate in at least two independent experiments.

XTT assay on *Candida* spp. biofilms in the course of maturation or already formed (24 h old)

The activity of samples on the metabolic activity of yeasts in biofilms at two different phases of development was assessed according to Millot et al (2017) with slight modifications.^[54] Yeasts were first grown for 48 h on SGC agar slants. Then, four loopfuls of this culture were carefully transferred to 30 mL of Yeast Nitrogen Base medium (Difco, Detroit, MI, USA), supplemented with 0.05 mol L⁻¹ glucose (Merck, Darmstadt, Germany) (YNB-Glc) and incubated overnight at 37 °C without shaking. This culture was then centrifuged at 3000 g for 10 min (SL40R centrifuge, Thermo scientific, Waltham, USA), washed twice in 0.1 M phosphate-buffered saline (PBS, pH 7.2, GIBCO, NY, USA), and adjusted to 2 × 10⁷ blastospores per mL in YNB-Glc. The yeasts suspension (200 µL) was dispensed to a sterile untreated flat-bottom 96-well polystyrene plate (Costar, Corning, USA) and incubated for 3 h at 37 °C corresponding to the adherent phase. The non-adherent yeasts were then removed by vacuum and adherent cells were washed once with 0.1 M PBS. For the antibiofilm test, an additional step was performed: after washing, 200 µL of YNB-Glc were added into each well which were incubated for 24 h at 37 °C and then washed again to remove the non-adherent cells of the mature biofilm. Therefore, after 3 h (for the antimaturation test) or 24 h of incubation (for the antibiofilm test), 100 µL of YNB-Glc were added as well as 100 µL of previously prepared samples. Indeed, during incubation steps, serial two-fold dilutions of extracts, Bh-L EO, and commercial compounds stock solutions were prepared in YNB-Glc at final concentrations ranging from 500 to 62.5 µg mL⁻¹, 1000 µg mL⁻¹ to 0.98 µg mL⁻¹ and 4 × 10⁻³ to 0.0039 × 10⁻³ mol L⁻¹, respectively (except for (±)-β-pinene: 6 × 10⁻³ to 0.006 × 10⁻³ mol L⁻¹) in other microplates. Some wells were reserved for controls, i.e., non-treated yeasts (negative control) and yeasts treated by DMSO 2%. The plates were incubated for 24 h at 37 °C. The wells were washed twice with PBS to remove free floating microorganisms and observed under an inverted optical microscope (IX51® inverted microscope, Olympus America Inc., Melville, NY, USA) prior to biofilm quantification using a metabolic assay based on the reduction of a tetrazolium salt (XTT). Briefly, 300 mg L⁻¹ XTT (Merck, Darmstadt, Germany) and 0.13 × 10⁻³ mol L⁻¹ menadione (Merck, Darmstadt, Germany) were added to 200 µL of PBS. Plates were incubated for 3 h at 37 °C without shaking. The absorbance at 492 nm was measured (Infinite M Plex absorbance reader, TECAN, Zürich, Switzerland). All conditions were performed quadruplicate in two independent experiments.

For each tested concentration of every sample, the inhibition percentage was calculated according to the following formula:

$$100 - \left[\left(100 \times X_{\text{absorbance of the treated group}} \right) / X_{\text{absorbance of control}} \right]$$

The IC₅₀ (concentration that inhibits 50% of the biofilm) was determined using a non-linear regression graph obtained with GraphPad Prism 8 software (GraphPad, San Diego, CA).

Crystal violet assay on 24 h old biofilm of dual-species *S. aureus*-*C. albicans*

Crystal violet (CV) assay was performed to assess the biomass of mono- and dual-species biofilms (*S. aureus* ATCC 29213 and/or *C. albicans* ATCC 28367) in untreated flat-bottom 96-well polystyrene plate (Costar) based on Hernandez-Cuellar *et al* (2022) with slight modifications.^[55] Yeasts were first grown for 48 h on SGC agar slants. *S. aureus* was grown for 48 h on brain heart infusion (BHI) (BD Difco™, Franklin Lakes, USA) agar plates at 37 °C. Then, each strain was cultured in liquid BHI medium at 37 °C overnight, with agitation of 80 rpm for *S. aureus* only. Twenty-five mL of these cultures were centrifuged at 5000 g (SL40R centrifuge) for 5 min. The pellet was washed twice with PBS. Cell concentration was determined by absorbance measurement at 600 nm for *S. aureus*, and by direct counting with a Fast-Read 102® counting chamber (Biosigma, Venice, Italy) for *C. albicans*. For mono-species biofilm, 200 µL of cultures at 10⁶ cells per mL were inoculated in each well. For dual-species biofilms, 100 µL of a 1:1 ratio of each suspension at 10⁶ cells/mL were inoculated. Microplate wells containing a mono- or dual-species suspension were incubated at 37 °C for 2 h. Non-adherent cells were removed by washing with PBS, 200 µL of BHI were added into each well and plates were incubated for an additional 22 h before being washed with PBS to remove planktonic cells. A serial two-fold dilution of the BHI-L EO stock solution was performed in BHI medium, the final concentrations ranging between 62.5 µg mL⁻¹ and 3.91 µg mL⁻¹. Those prepared BHI-L EO samples were then transferred into the plate containing mono or dual species biofilm. Some wells without treatment or treated by DMSO 2% or with medium only were preserved. The plates were incubated for 24 h at 37 °C. Wells were then washed with 200 µL of PBS to remove planktonic cells, fixed with methanol (15 min), and stained with 0.9% CV dye (5 min). Afterward, the excess colorant was discarded, and the plates were rinsed with distilled water. To quantify the biofilm, 200 µL of a 33% solution of acetic acid was added per well (20 min incubation), and the content of each well was transferred to a new microplate before measuring the absorbance at 510 nm (Infinite M Plex absorbance reader, TECAN, Zürich, Switzerland). The absorbance value of the medium control was subtracted from that obtained for each well. The percentage of biofilm reduction was calculated by the following equation:

Biofilm reduction (%) =

$$100 - \left[\left(100 \times X_{\text{absorbance of the treated group}} \right) / X_{\text{absorbance of control}} \right]$$

All conditions were performed in triplicate in three independent experiments.

Cytotoxic assay

Cytotoxicity of compound solutions was determined on HeLa human epithelial cervix cells using the XTT cell viability assay, which measures cellular metabolic activity, as previously described.^[56] HeLa cells were cultured as monolayers at 37 °C in a 5% CO₂-95% air humidified atmosphere in Dulbecco's minimum essential medium (DMEM) (Merck, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS). Cells (15000 cells per well) were seeded into each well of 96-well plates and cultured until

approximately 80% confluence before being treated by various concentrations of compounds solution (50% biofilm inhibitory concentration (IC_{biofilm50}), 2-fold IC_{biofilm50}, and 4-fold IC_{biofilm50}) for 24 h. Cell viability was assessed using the cell proliferation kit II (XTT, RocheDiagnostics, Rotkreuz, Switzerland) according to the manufacturer's protocol. Cells treated by DMSO 2% or with medium only were used as controls. Absorbance [A492 nm–A690 nm] was measured to quantify viable cells spectrophotometrically. Cytotoxicity percentages were determined as [1-(Absorbance mean of treated group/Absorbance mean of control)]×100. Calculations were done using the GraphPad Prism 8 software (GraphPad, San Diego, CA). All experiments were performed at least in triplicate in two independent experiments.

Statistical analyses

Analyses were performed using the GraphPad Prism version 8 software (GraphPad softwareInc., La Jolla, CA, USA). The Kruskal-Wallis test and post hoc Dunn's multiple comparisons test were performed to measure the value difference between untreated and treated samples. *p*-Values less than or equal to 0.05 were considered statistically significant.

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Author Contributions

S. D., J. D. performed the experiments; S. D., J. D., C. B., J. L., M., T. N., C. I., M. G. analyzed the data; S. D., C. I., M. G. wrote the article; G. H., C. B., J. L. contributed materials tools; C. B., J. L., C. I., M. G. conceived and designed the experiments. All authors have read and approved the final version of the manuscript.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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